



## Anti-inflammatory functions of glucocorticoid-induced genes

Andrew R. Clark

### ► To cite this version:

Andrew R. Clark. Anti-inflammatory functions of glucocorticoid-induced genes. *Molecular and Cellular Endocrinology*, 2007, 275 (1-2), pp.79. <10.1016/j.mce.2007.04.013>. <hal-00531924>

**HAL Id: hal-00531924**

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Submitted on 4 Nov 2010

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HAL Authorization

## Accepted Manuscript

Title: Anti-inflammatory functions of glucocorticoid-induced genes

Author: Andrew R. Clark

PII: S0303-7207(07)00184-0  
DOI: doi:10.1016/j.mce.2007.04.013  
Reference: MCE 6649

To appear in: *Molecular and Cellular Endocrinology*

Received date: 9-3-2007  
Accepted date: 25-4-2007

Please cite this article as: Clark, A.R., Anti-inflammatory functions of glucocorticoid-induced genes, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2007.04.013

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**Anti-inflammatory functions of glucocorticoid-induced genes.**

Andrew R. Clark

Kennedy Institute of Rheumatology Division

Imperial College London

1 Aspenlea Road

Hammersmith

London W6 8LH

United Kingdom

Email [andy.clark@imperial.ac.uk](mailto:andy.clark@imperial.ac.uk)

Phone +44 208 383 4430

Fax +44 208 383 4496

**Abbreviations:** AnxA1, annexin A1; AP1, activating protein 1; ARE, adenylate/uridylate-rich element; AREBP, ARE binding protein; C/EBP, CCAAT/enhancer binding protein; CCL, chemokine (C-C motif) ligand; CLE0, conserved lymphokine element 0; COX-2, cyclooxygenase 2; CSF2, colony stimulating factor 2; CXCL, chemokine (C-X-C motif) ligand; dc, dendritic cell; Dok-1, downstream of tyrosine kinase 1; DUSP, dual specificity phosphatase; ENaC, epithelial sodium channel; ERK, extracellular signal regulated kinase; Fox, forkhead box transcription factor; FPR, formyl peptide receptor; GC, glucocorticoid; GILZ, GC-induced leucine zipper protein; GR, GC receptor; GR<sup>dim</sup>, dimerization defective GR mutant; GRE, GC response element; ICAM, intracellular adhesion molecule; IκB, inhibitor of NFκB; IL, interleukin; LZ, leucine zipper; MAPK, mitogen activated protein kinase; MKP, MAPK phosphatase; NFκB, nuclear factor of κB; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol 3 kinase; RA, rheumatoid arthritis; siRNA, short interfering RNA; SLAP, src-like adaptor protein; STAT, signal transducer and activator of transcription; TAT, tyrosine aminotransferase; TNF, tumor necrosis factor; TTP, tristetraprolin; UTR, untranslated region

**ABSTRACT.**

There is a broad consensus that glucocorticoids (GCs) exert anti-inflammatory effects largely by inhibiting the function of nuclear factor  $\kappa$ B (NF $\kappa$ B) and consequently the transcription of pro-inflammatory genes. In contrast, side-effects are thought to be largely dependent on GC-induced gene expression. Biochemical and genetic evidence suggests that the positive and negative effects of GCs on transcription can be uncoupled from one another. Hence, novel GC-related drugs that mediate inhibition of NF $\kappa$ B but do not activate gene expression are predicted to retain therapeutic effects but cause fewer or less severe side effects. Here we critically re-examine the evidence in favor of the consensus, binary model of GC action and discuss conflicting evidence, which suggests that anti-inflammatory actions of GCs depend on the induction of anti-inflammatory mediators. We propose an alternative model, in which GCs exert anti-inflammatory effects at both transcriptional and post-transcriptional levels, both by activating and inhibiting expression of target genes. The implications of such a model in the search for safer anti-inflammatory drugs are discussed.

**INTRODUCTION.**

The powerful anti-inflammatory actions of glucocorticoid hormones (GCs) were discovered in the late 1940s by Philip Hench and his collaborators, who were attempting to treat the chronic and debilitating inflammatory disease, rheumatoid arthritis (RA). Within little more than a year the newly isolated GC cortisone had been licensed by the United States Food and Drug Administration, several thousand RA patients around the world had been treated with it, and the Nobel Prize for Physiology or Medicine (1950) had been awarded to Dr Hench and his collaborators, Tadeus Reichstein and Edward Kendall. In large part, this reflects the paucity of anti-inflammatory treatments then available, and the almost miraculous beneficial effects of cortisone. Since that time synthetic GCs have become a mainstay in the treatment of diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, multiple sclerosis and many others. However, severe side-effects of GCs have been recognised from the very beginning, and were even detailed in Philip Hench's Nobel prize lecture (Hench, 1950). For decades, clinicians and basic scientists have been fascinated by GCs because they are at the same time amongst the most useful and the most problematic compounds in the pharmacopoeia. If their desired anti-inflammatory effects could somehow be uncoupled from their side effects the clinical benefits would be enormous. It has become widely accepted that such uncoupling might be achieved through separation of activatory and inhibitory effects of glucocorticoids on gene expression. Here this supposition will be re-examined from a critical viewpoint, which emphasizes the gaps in our current knowledge of GC action and the potential roles of GC-induced genes in the inhibition of inflammation.

**ANTI-INFLAMMATORY ACTION OF GLUCOCORTICOIDS: THE CURRENT PARADIGM.**

Effects of GCs on gene expression are mediated by the GC receptor (GR), a member of a large family of nuclear hormone receptor transcription factors (Newton, 2000; Rhen and Cidlowski, 2005). When activated by ligand, GR is released from a multiprotein complex in the cytoplasm and translocates to the nucleus. Once there it can positively regulate gene expression by dimerizing and binding to palindromic GC response elements (GREs) in the promoters of target genes. Such a sequence exists in the promoter of tyrosine aminotransferase (TAT), a metabolic enzyme. Disturbances of metabolism are amongst the important side effects of prolonged treatment with synthetic GCs, and may be caused by the upregulation of TAT and other genes such as phosphoenolpyruvate carboxykinase (PEPCK). GR can also interact in a dimerization-independent manner with other transcription factors such as nuclear factor  $\kappa$ B (NF $\kappa$ B) and activating protein 1 (AP1), impairing their ability to activate gene expression, a process known as transrepression (throughout this article, “transrepression” is used in the strict sense of an inhibitory process involving direct or indirect physical interaction of GR with a target transcription factor). The molecular details of this mechanism are hotly debated as described elsewhere in this issue, and do not need to be explored again here. Most importantly, transrepression is both rapid and direct (mediated by pre-existing factors). NF $\kappa$ B is activated by most pro-inflammatory stimuli and, although its role is not restricted to the inflammatory response, it is important for the expression of many inflammatory mediators (Doyle and O'Neill, 2006; Grivennikov et al., 2006). For this reason its inhibition is, in principle, a powerful anti-inflammatory mechanism. Transrepression of AP1 may also contribute to the inhibition of anti-inflammatory gene expression, but its mechanism does not appear to be identical (Bladh et al., 2005).

The dimerization of GR is dependent on a short stretch of five amino acids known as the D loop, lying within the second zinc finger (Dahlman-Wright et al., 1993; Dahlman-Wright et al., 1991). Mutation of these residues strongly impaired (but did not abolish) binding of GR as a dimer to simple palindromic GREs in vitro. Mutation of alanine 458 to threonine within the D loop created a mutant (known as GR<sup>dim</sup>), which poorly activated GRE-dependent reporters but retained the ability to repress AP1-dependent (Heck et al., 1997; Heck et al., 1994) or NFκB-dependent reporters (Reichardt et al., 2001). In an elegant series of experiments, a mouse “knock-in” strain was generated, replacing endogenous GR with the GR<sup>dim</sup> mutant (Reichardt et al., 1998; Reichardt et al., 2001; Tuckermann et al., 1999). In macrophages or T cells expressing only GR<sup>dim</sup>, the synthetic GC dexamethasone (dex) retained its ability to suppress tumor necrosis factor (TNF), interleukin (IL)-1β, IL-2, IL-6 and cyclooxygenase 2 (COX-2) gene expression (Reichardt et al., 2001). Dex exerted comparable therapeutic effects on PMA-induced skin inflammation or LPS-induced endotoxemia in wild type and mutant mice. On the other hand TAT and PEPCK were poorly induced by dex in the GR<sup>dim</sup> mouse.

To a great extent these observations form the basis of the current consensus: that anti-inflammatory effects of GCs are largely mediated by transrepression, whereas side effects are largely mediated by activation of gene expression. From this premise it follows that uncoupling of the gene-activating and gene-suppressing properties of GR should yield clinical benefits. In other words “dissociated” GCs that selectively mediate transrepression rather than transactivation should show equivalent anti-inflammatory properties whilst causing less severe side effects (Belvisi et al., 2001; Rosen and Miner, 2005; Schacke et al., 2005). A simple course of action suggests itself for the discovery of dissociated GCs. Candidate compounds could be tested for their ability to inhibit a reporter controlled by multiple NFκB sites and to activate a reporter controlled by multiple GREs. Successful compounds should, of



course, pass the first test and fail the second. First generation dissociated GCs have been identified in this manner (Heck et al., 1997; Vanden Berghe et al., 1999; Vayssiere et al., 1997).

## RESERVATIONS.

### The complexity of GC action.

An important concern is that the standard model does poor justice to the great complexity of transcriptional control by GR. In the first place, the majority of genes directly activated by GCs do not appear to be controlled through conventional GREs. For example a microarray-based study of murine T lymphoma cells identified 44 direct GC targets (ie genes whose upregulation by GC was not prevented by cycloheximide). Of these, only five contained matches to the GRE consensus within 8 kb of the transcription start site (Chen et al., 2003). On the other hand, an *in silico* approach identified 565 exact matches to the GRE consensus in the human genome (Horie-Inoue et al., 2006). Of these, only 26 were located within 10 kb of transcribed genes. In DU145 (prostate cancer) cells chromatin immunoprecipitation analysis showed association of GR with only two of these putative regulatory sites, but the adjacent genes were not upregulated by GC. These investigations, unbiased by prior expectation, suggest that GCs tend to regulate gene expression through sequence elements that are not classical, consensus GREs.

Typically, GC responsive regions of *bona fide* GC-regulated genes are rather extended and contain binding sites for multiple transcription factors but only poor matches to the GRE consensus (Wang et al., 2004). A good example is the PEPCK gene, which contains two GR binding sites with limited homology to the GRE consensus. Efficient induction of gene expression by GCs requires binding of additional transcription factors to accessory sites within an extended, composite response element (Cassuto et al., 2005; Imai et al., 1990; Mitchell et al., 1994; Yamada et al., 1999). This complex element mediates the coordinate regulation of PEPCK expression by several agonists, including GCs,

insulin and retinoic acid. Composite GC responsive regions have been described in the promoters of tyrosine aminotransferase (Grange et al., 2001; Grange et al., 1989; Roux et al., 1995; Sassi et al., 1998) proliferin (Diamond et al., 1990),  $\alpha$ 1-acid glycoprotein (Alam et al., 1993; Nishio et al., 1993; Savoldi et al., 1997), the cyclin-dependent kinase inhibitor p21<sup>waf1/cip1</sup> (Cha et al., 1998; Cram et al., 1998),  $\beta$ 1-adrenergic receptor (Tseng et al., 2001), glucose-6-phosphate transporter (Kallwellis-Opara et al., 2003), pyruvate dehydrogenase kinase 4 (Kwon et al., 2004), monoamine oxidase (Manoli et al., 2005; Ou et al., 2006),  $\beta$ -casein (Rosen et al., 1998; Wyszomierski and Rosen, 2001) and others. Although GR alone has low affinity for sites that diverge from the GRE consensus, high affinity binding and transcriptional activation can be restored in the presence of multiple sub-optimal GR binding sites such as half GREs (Lechner et al., 1997) or adjacent binding sites for other transcription factors (Guido et al., 1996; Morin et al., 2000; Stafford et al., 2001; Strahle et al., 1988). Recruitment to such sites is thought to be mediated by both protein-DNA interactions and protein-protein interactions of GR with other transcription factors. GR has been shown to cooperate with transcription factors of many different classes, including the zinc finger transcription factor, stimulatory protein 1 (Sp1) (Manoli et al., 2005; Marinovic et al., 2002; Ou et al., 2006), homeobox proteins (Subramaniam et al., 2003), ets-related proteins (Aittomaki et al., 2000; Aurrekoetxea-Hernandez and Buetti, 2004; Mullick et al., 2001), interferon response factors (Jiang et al., 2004), helix-loop-helix factors (Jiang et al., 2004; Tseng et al., 2001), members of the CCAAT/enhancer-binding protein (C/EBP), forkhead box (Fox) and signal transducer and activator of transcription (STAT) families, and even NF $\kappa$ B itself (Hofmann and Schmitz, 2002).

Functional interactions of GR with members of the STAT family are well reviewed elsewhere (Rogatsky and Ivashkiv, 2006). The STATs mediate transcriptional activation in response to cytokine signalling, and exert pleiotropic effects on immunity and inflammation (Brierley and Fish, 2005).

Expression of  $\beta$ -casein is synergistically regulated by prolactin-activated STAT5 and GC-activated GR (Kabotyanski et al., 2006; Lechner et al., 1997; Stocklin et al., 1996; Stoecklin et al., 1999; Wyszomierski and Rosen, 2001). The latter regulates transcription via multiple GRE half-sites, and is dependent on STAT5 for recruitment to the  $\beta$ -casein 5' region. A functional interaction between GR and STAT5 is also implicated in the cooperative regulation of toll-like receptor 2 (TLR2) expression by GC and TNF (Hermoso et al., 2004). In liver, GR is recruited to STAT5 regulated genes such as insulin-like growth factor 1, and is required for normal post-natal growth. Importantly, this GR-mediated transcriptional control is intact in liver cells expressing only GR<sup>dim</sup> (Tronche et al., 2004). In a similar fashion, STAT3 and GR can physically interact and cooperate to activate promoters containing either STAT or GR binding sites (De Miguel et al., 2003; Kordula and Travis, 1996; Lerner et al., 2003; Takeda et al., 1998; Zhang et al., 1997).

Three distinct mechanisms of functional interaction between GR and members of the C/EBP family of transcription factors have been described. Least direct of all, GCs can upregulate expression of C/EBPs, for example in muscle (Penner et al., 2002; Yang et al., 2005), liver (Cram et al., 1998; Gotoh et al., 1997; Matsuno et al., 1996; Ramos et al., 1996) or differentiating adipocytes (Hernandez et al., 2003; Shi et al., 2000). Secondly, GR can cooperate with C/EBP transcription factors to activate transcription through composite response elements such as those of  $\alpha$ 1 acid glycoprotein (Alam et al., 1993; Chang et al., 1998; Savoldi et al., 1997), PEPCK (Arizmendi et al., 1999; Crosson and Roesler, 2000; Yamada et al., 1999) and  $\beta$  casein genes (Wyszomierski and Rosen, 2001). Finally, GCs can enhance the function of C/EBP $\beta$ . This process involves the phosphorylation of C/EBP $\beta$  and exchange of associated coactivator/corepressor proteins, although it is unclear whether a direct interaction between GR and C/EBP $\beta$  is required (Berg et al., 2005; Boruk et al., 1998; Wiper-Bergeron et al., 2003).

Several members of the Fox family of transcription factors have been shown to interact functionally with GR. Such interactions may be particularly significant to the regulation of carbohydrate metabolism, where GCs and insulin have opposing actions. Glucose 6 phosphatase (Nakae et al., 2001) pyruvate dehydrogenase kinase 4 (Furuyama et al., 2003; Kwon et al., 2004), glucose 6 phosphate transporter (Kallwellis-Opara et al., 2003) and insulin-like growth factor binding protein 1 (Yeagley et al., 2001) are cooperatively regulated by GR and FoxO transcription factors. Insulin causes inactivation of FoxOs via Akt-mediated phosphorylation, thereby antagonizing GC-induced gene expression. A similar mechanism may be relevant to the anti-inflammatory action of GCs (Asselin-Labat et al., 2005). IL2-induced, Akt-mediated phosphorylation of FoxO3 antagonizes the induction by GCs of glucocorticoid-induced leucine zipper (GILZ; see below).

### **Post-transcriptional gene regulation by GCs.**

Endogenous GCs are upregulated by pro-inflammatory cytokines and play a critical role in the resolution of inflammation. To fulfil this function they must be able to suppress ongoing inflammatory responses. Transrepression is potentially a powerful mechanism for inhibiting inflammation, but an important limitation is that it can be effective only if GCs are present during the period of active transcription. For many inflammatory mediators the activation of transcription is highly transient and this window of opportunity is correspondingly narrow. In experimental settings GCs are capable of blocking gene expression even if added some time after a pro-inflammatory stimulus, whilst inhibitors of transcription are ineffective (Chivers et al., 2006; Lasa et al., 2002; Newton et al., 1998). There must therefore exist suppressive mechanisms that function at a post-transcriptional level. By definition these have nothing to do with transrepression, which can have no influence on pre-existing mRNAs.

In fact GCs have long been known to inhibit inflammatory gene expression at a post-transcriptional level, via destabilisation of mRNA or inhibition of translation (Ing, 2005; Stellato, 2004). Targets of such regulation include COX-2 (Chivers et al., 2006; Lasa et al., 2001; Newton et al., 1998; Ristimäki et al., 1996), TNF (Han et al., 1990; Kontoyiannis et al., 1999; Swantek et al., 1997), vascular endothelial growth factor (Gille et al., 2001), interferon  $\beta$  (Peppel et al., 1991), colony stimulating factor 2 (CSF2) (Bergmann et al., 2004; Tobler et al., 1992; Tran et al., 2005), IL-5 (Staples et al., 2003), IL-6 (Amano et al., 1993; Tobler et al., 1992), IL-1 $\alpha$  and -1 $\beta$  (Amano et al., 1993; Lee et al., 1988), inducible nitric oxide synthase (Korhonen et al., 2002) and several chemokines (Berkman et al., 1995; Brach et al., 1992; Chang et al., 2001; Chaudhary and Avioli, 1996; Chivers et al., 2006; Mukaida et al., 1991; Poon et al., 1999; Stellato et al., 1999; Tobler et al., 1992). The majority of these mRNAs have in common the presence of adenylate/uridylate-rich elements (AREs) in their 3' untranslated regions (UTRs). AREs form specific binding sites for ARE-binding proteins (AREBPs) such as tristetraprolin (TTP), which function by recruiting components of the cellular mRNA degradation machinery and causing rapid destruction of target mRNAs (Carrick et al., 2004; Fenger-Gron et al., 2005; Guhaniyogi and Brewer, 2001; Kracht and Saklatvala, 2002; Lykke-Andersen and Wagner, 2005; Wilusz et al., 2001). ARE-AREBP complexes also mediate the post-transcriptional regulation of mRNA stability by signal transduction pathways. For example many ARE-containing inflammatory mRNAs are unstable in the absence of an appropriate stimulus, but are transiently stabilized following the activation of the p38 mitogen-activated protein kinase (MAPK) pathway (Clark et al., 2003; Dean et al., 2004), which brings about the phosphorylation and inactivation of TTP (Stoecklin et al., 2004). An unusual mechanism exists for the GC-induced destabilization of CCL2 (chemokine C-C motif ligand 2) mRNA. GCs can destabilize this transcript independently of ongoing transcription and translation via an element in the 5' UTR (Poon et al., 1999), which forms a direct target for ligand-activated GR (Dhawan et al., 2007). In almost every other case, post-transcriptional

effects of GCs on inflammatory gene expression are dependent on GC-induced gene expression. Therefore, in classical chase experiments to study mRNA stability, effects of GCs can be masked unless they are present for some time prior to the addition of the transcriptional inhibitor actinomycin D. As described below, GCs may influence pro-inflammatory gene expression by altering the expression of AREBPs, by modulating the activity of the signalling pathways that control them, or both. Post-transcriptional effects of GCs are easily overlooked, cannot be explained in terms of transrepression and scarcely feature in most reviews of GC action. However, a recent microarray-based study of global changes in gene expression suggests that such effects may account for at least 50% of GC-mediated changes in steady-state mRNA levels (Fan et al., 2006).

### **Evidence in favour of transrepression.**

It is often stated as fact that GCs inhibit pro-inflammatory gene expression principally by means of transrepression. So much depends on this assertion that it is worth re-examining its basis in evidence. The most important supporting observation is that some anti-inflammatory effects of GCs are preserved in mice that express only a dimerization-defective form of GR (Reichardt et al., 2001). An important unanswered question is whether GC responses of the GR<sup>dim</sup> mouse remain intact in more complex models of inflammatory disease than PMA-induced skin inflammation or LPS-induced endotoxemia. Another question is whether GR-mediated activation of gene expression is completely abolished by the mutation of a single amino acid within the GR dimerization domain. In transfected cells GR<sup>dim</sup> is capable of inducing at least some GC target genes (Rogatsky et al., 2003), cooperative gene regulation by GCs and STAT5 is spared in the GR<sup>dim</sup> mouse (Tronche et al., 2004), and at least one anti-inflammatory mediator is effectively induced by GCs in murine macrophages expressing only GR<sup>dim</sup> (Abraham et al., 2006). In the absence of microarray data describing global transcriptional changes in cells expressing GR<sup>dim</sup>, it is too early to conclude how broadly and how strongly the induction of gene expression by GCs is impaired, therefore it remains a strong possibility that this mutation does not

make a clear distinction between gene activatory and gene-suppressive effects of GCs *in vivo*. Other than the GR<sup>dim</sup> mouse, we know of no murine genetic model supporting the assertion that transrepression is the only (or even principal) anti-inflammatory mechanism of GCs.

There is firm evidence that GR can transrepress NFκB, AP1 and other transcription factors, and plentiful, though sometimes contradictory, evidence concerning the molecular mechanisms involved. Neither of these constitutes proof that transrepression is the principal mechanism by which GCs inhibit pro-inflammatory gene expression. Several different elements would need to be established in the hypothetical case of a gene containing an NFκB site in its promoter. IL-8 (otherwise known as chemokine C-X-C motif ligand 8, CXCL8) is an archetypal GC-sensitive pro-inflammatory gene that is considered a target of transrepression, and is discussed as an illustrative example where appropriate.

*GCs inhibit gene expression at the transcriptional level but not at the post-transcriptional level.* Many pro-inflammatory mediators (including CXCL8) have both NFκB sites in their promoters and AREs in their 3' UTRs, and can be suppressed by GCs at both transcriptional and post-transcriptional levels. However, GC-induced changes in pro-inflammatory mRNA levels are often ascribed to transcriptional effects alone without formal supporting evidence, for example nuclear run-on and actinomycin D chase experiments. Transcriptional and post-transcriptional mechanisms are in no way mutually exclusive, and some individual genes have been shown to be suppressed by GCs at both levels in a single cell type (Lee et al., 1988; Newton et al., 1998; Stellato et al., 1999; Zitnik et al., 1994). In other cases data on the relative contributions of transcriptional and post-transcriptional mechanisms are contradictory. Dex did not strongly inhibit CXCL8 transcription in nuclear run-on experiments, but potently destabilized CXCL8 mRNA in bone marrow stromal cells, lung fibroblasts, epithelial cells or cell lines (Chang et al., 2001; Chaudhary and Avioli, 1996; Chivers et al., 2006; Tobler et al., 1992). In contrast, other

reports described dex-mediated inhibition of an CXCL8 promoter construct and a lack of effect of dex on CXCL8 mRNA stability (Edwards et al., 2005; Kwon et al., 1994).

*Inhibitory effects of GC do not require de novo gene expression.* In its strictest sense, transrepression is a rapid and direct mechanism that is mediated by pre-existing factors and does not require *de novo* gene expression. Consistent with this, the protein synthesis inhibitor cycloheximide (CHX) did not diminish the inhibitory effect of dex on expression of IL-6, intracellular adhesion molecule (ICAM)-1 or COX-2 mRNAs (De Bosscher et al., 1997; Wissink et al., 1998). In other studies, CHX blocked the GC-mediated suppression of urokinase plasminogen activator, COX-2 and CXCL8 mRNA (Chang et al., 2001; Chaudhary and Avioli, 1996; Chivers et al., 2006; Henderson and Kefferd, 1993; Newton et al., 1998; Tobler et al., 1992). This implies that GC-induced gene expression is involved, and that transrepression is insufficient for gene suppression in some circumstances.

*GC-dependent inhibition of gene expression is not accompanied by a global defect in NF $\kappa$ B activation.* Whereas other models invoke general inhibition of nuclear translocation or DNA binding of NF $\kappa$ B, the transrepression model states that GCs inhibit NF $\kappa$ B function at a level downstream of DNA binding. In some studies anti-inflammatory effects of GCs were not accompanied by changes in DNA-binding activity of NF $\kappa$ B as measured by electrophoretic mobility shift assay (Brostjan et al., 1996; De Bosscher et al., 1997; Hofmann et al., 1998; Nissen and Yamamoto, 2000; Ray et al., 1997; Wissink et al., 1998). However, in other cases GCs were found to inhibit NF $\kappa$ B activation as measured by the same assay (Eberhardt et al., 2002; Goppelt-Struebe et al., 2000; Kurata and Yamamoto, 1999; Kurokouchi et al., 2000; Ma et al., 2004; Mukaida et al., 1994; Vital et al., 2003). The latter observations are inconsistent with the transrepression model.

*GCs do not inhibit the recruitment of NF $\kappa$ B to the target promoter in vivo.* Subtle variations in NF $\kappa$ B core binding sequence and flanking residues can have a profound effect on regulatory activities (Doyle and O'Neill, 2006; Hoffmann et al., 2003; Leung et al., 2004), and binding of NF $\kappa$ B to sites in



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chromatin may not be accurately reflected by binding to oligonucleotide probes *in vitro* (Grivennikov et al., 2006; Saccani et al., 2002). For these reasons it is important to show that GCs do not impair the interaction of NFκB with the relevant cognate site *in vivo*, an important proposition of the transrepression model. Accordingly, GCs did not inhibit binding of NFκB to the 5' regions of the chemokines CXCL1 and CXCL9 (Ogawa et al., 2005), ICAM-1 (Liden et al., 2000) or CXCL8 (De Bosscher et al., 2005; Luecke and Yamamoto, 2005; Nissen and Yamamoto, 2000). To our knowledge, effects of GCs on NFκB recruitment at other transrepressed genes have not been reported.

*GC-dependent inhibition of gene expression is accompanied by recruitment of GR to the target promoter.* Another central element of the transrepression model is that physical (direct or indirect) interaction between GR and NFκB mediates recruitment of GR to inflammatory promoters. Such recruitment has been demonstrated in very few examples, one of which is CXCL8 (Garside et al., 2004; Luecke and Yamamoto, 2005; Nissen and Yamamoto, 2000). In HeLa cells dexamethasone promoted recruitment of GR to the CXCL8 promoter and inhibited TNF-induced expression of CXCL8. In contrast the antagonistic ligand RU486 did neither (Garside et al., 2004), suggesting a causal link between GR recruitment and gene suppression. However, in at least one instance recruitment of GR to an inflammatory promoter occurred in the absence of transrepression (Luecke and Yamamoto, 2005; Nissen and Yamamoto, 2000). This observation raises the question of whether the recruitment of GR to NFκB sites is both necessary and sufficient for inhibition of gene expression.

## **GC-INDUCED ANTI-INFLAMMATORY MEDIATORS.**

### **Inhibitor of NFκB α (IκBα).**

In the absence of appropriate stimuli, NFκB components are held in the cytoplasm by interactions with inhibitors of NFκB (IκBs). Pro-inflammatory signaling pathways cause the degradation of IκB proteins and the release of NFκB, which then enters the nucleus to activate target genes. Two groups

independently identified the upregulation of I $\kappa$ B $\alpha$  as a putative mechanism by which GCs could impair NF $\kappa$ B function and inhibit expression of immune or inflammatory mediators (Auphan et al., 1995; Scheinman et al., 1995a; Scheinman et al., 1995b). A number of problems with this hypothesis have since been raised. In several different cell types the GC-induced upregulation of I $\kappa$ B $\alpha$  either did not occur or was not sufficient to alter the degradation of I $\kappa$ B $\alpha$  protein, and the nuclear translocation of NF $\kappa$ B in response to pro-inflammatory stimuli (Adcock et al., 1999; Adcock et al., 1997; Auwardt et al., 1998; Brostjan et al., 1996; De Bosscher et al., 1997; Hofmann et al., 1998; Liden et al., 2000; Nissen and Yamamoto, 2000; Pruett et al., 2003; Ray et al., 1997; Wissink et al., 1998). As described above, inhibition of inflammatory gene expression could occur without apparent changes in NF $\kappa$ B promoter occupancy (Liden et al., 2000; Luecke and Yamamoto, 2005). Repression of NF $\kappa$ B function was not dependent on de novo gene expression {De Bosscher et al., 1997; Nissen and Yamamoto, 2000; Ogawa et al., 2005; Wissink et al., 1998). Novel GR agonists have been described, which enhanced I $\kappa$ B $\alpha$  expression but did not exert anti-inflammatory effects (Heck et al., 1997). GCs efficiently repressed a chimeric transcription factor consisting of the yeast Gal4 DNA binding domain and the transcriptional activation domain of the NF $\kappa$ B subunit p65 (De Bosscher et al., 1997; De Bosscher et al., 2005; Vanden Berghe et al., 1999). The nuclear localization and DNA binding activity of this fusion protein were not regulated by GCs.

All of these observations clearly establish that inhibition of NF $\kappa$ B function *can* occur independently of I $\kappa$ B $\alpha$  induction. However, they fall short of proving that I $\kappa$ B $\alpha$  induction plays no part in the anti-inflammatory actions of GCs. I $\kappa$ B $\alpha$  expression is tissue-specifically regulated, and is enhanced by GCs in several primary or transformed cell types (Almon et al., 2005; Auphan et al., 1995; Kang et al., 2006; Kurata and Yamamoto, 1999; Kurokouchi et al., 2000; Quan et al., 2000; Ramdas and Harmon, 1998; Scheinman et al., 1995a; Shames et al., 1998; Stojadinovic et al., 2006; Thiele et al., 1999). In

some cases GC treatment inhibited I $\kappa$ B $\alpha$  degradation, nuclear translocation of p65 or formation of nuclear NF $\kappa$ B complexes (Eberhardt et al., 2002; Goppelt-Strube et al., 2000; Kurata and Yamamoto, 1999; Kurokouchi et al., 2000; Ma et al., 2004; Mukaida et al., 1994; Vital et al., 2003). This is not consistent with a model in which GCs inhibit NF $\kappa$ B only at a level downstream of DNA binding, although it does not conclusively prove that upregulation of I $\kappa$ B $\alpha$  is critical. GR-mediated inhibition of NF $\kappa$ B function through protein-protein interactions (classical transrepression) and through upregulation of inhibitory molecules such as I $\kappa$ B $\alpha$  are, of course, not mutually exclusive mechanisms. I $\kappa$ B $\alpha$ -mediated inhibitory effects of GC may be significant in particular tissues, and in the context of prolonged GC exposure. Mice lacking I $\kappa$ B $\alpha$  have a severe inflammatory phenotype and die soon after birth (Beg et al., 1995; Chen et al., 2000; Klement et al., 1996), which is likely to complicate experiments to directly address the role of I $\kappa$ B $\alpha$  in responses to GCs. The question might be best answered by studying GC responses in conditional and/or tissue-specific I $\kappa$ B $\alpha$  knockouts.

The I $\kappa$ B $\alpha$  promoter does not possess a typical palindromic GRE (Deroo and Archer, 2001) but does have a potential half-site located between two NF $\kappa$ B elements (Heck et al., 1997). Dex induced only a twofold increase in I $\kappa$ B $\alpha$  mRNA and protein in wild-type fibroblasts, and this increase did not occur in fibroblasts expressing GR<sup>dim</sup> (Reichardt et al., 2001). The implication is that upregulation of I $\kappa$ B $\alpha$  is dependent on dimerization of GR, although the detailed mechanism of gene regulation remains to be established.

## DUSP1

Dual specificity phosphatase 1 (DUSP1) is the first described member of a large family of phosphatases that catalyse removal of phosphate from serine, threonine or tyrosine residues (Dickinson and Keyse, 2006; Liu et al., 2007; Theodosiou and Ashworth, 2002). It is expressed in response to GCs

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in a wide variety of cell types including mast cells (Kassel et al., 2001), monocytes or macrophages (Abraham et al., 2006; Aeberli et al., 2006; Bhattacharyya et al., 2007; Chen et al., 2002; Zhao et al., 2005), microglia (Zhou et al., 2007), T lymphocytes (Li et al., 2006), dermal, lung and synovial fibroblasts (Phillips et al., 2006; Toh et al., 2004; Yang et al., 2006), endothelial cells (Furst et al., 2007), osteoblasts (Engelbrecht et al., 2003; Leclerc et al., 2004), keratinocytes (Onda et al., 2006; Stojadinovic et al., 2006), adipocytes (Bazuine et al., 2004), lung epithelial cells (Chivers et al., 2006; Hermoso et al., 2004), airway smooth muscle cells (Issa et al., 2007) and HeLa cells (Imasato et al., 2002; Lasa et al., 2002). Typically expression is quite rapid (within one hour), sustained (up to 24 hours), requires relatively low concentrations of GC, and is blocked by the GR antagonist RU486. In transfected COS7 cells a murine DUSP1 promoter construct was activated by dex in a manner dependent on GR dimerization (Kassel et al., 2001). However, the induction of DUSP1 by dex was unimpaired in mouse macrophages expressing only GR<sup>dim</sup> (Abraham et al., 2006), implying that GR dimerization is unnecessary. The precise mechanism of regulation of DUSP1 by GCs remains to be determined.

DUSP1 is otherwise known as MAPK phosphatase 1 (MKP-1) because it dephosphorylates and inactivates members of the mitogen-activated protein kinase (MAPK) family. Preferred targets are cJun N-terminal kinase (JNK) and p38 MAPK (Franklin and Kraft, 1997), although GC-induced expression of DUSP1 has also been linked to inhibition of the extracellular signal-regulated kinase (ERK) pathway in certain cell types (Kassel et al., 2001; Wu, Roth et al., 2006). The substrate selectivity of DUSP1 is not fully understood (Abraham and Clark, 2006). The MAPKs regulate inflammatory gene expression at both transcriptional and post-transcriptional levels via the phosphorylation of transcription factors and RNA binding proteins (Clark et al., 2003; Dean et al., 2004; Ip and Davis, 1998; Kracht and Saklatvala, 2002; Ono and Han, 2000). DUSP1 is induced by pro-inflammatory stimuli and forms a negative feedback loop to limit MAPK signaling and the expression of inflammatory mediators. Hence

overexpression of DUSP1 attenuates signaling of the JNK and p38 MAPK pathways and inhibits the expression of several inflammatory genes (Chen et al., 2002; Nimah et al., 2005; Zhao et al., 2005). Conversely, DUSP1<sup>-/-</sup> macrophages show abnormally prolonged activation of JNK and p38, which results in overexpression of inflammatory genes (Chi et al., 2006; Hammer et al., 2006; Salojin et al., 2006; Zhao et al., 2006). In LPS-induced endotoxemia and collagen-induced arthritis (experimental models of acute or chronic inflammation, respectively), DUSP1<sup>-/-</sup> mice show exaggerated responses (Chi et al., 2006; Hammer et al., 2006; Salojin et al., 2006; Zhao et al., 2006). Hence DUSP1 is an important negative regulator of inflammatory responses (Abraham and Clark, 2006; Dickinson and Keyse, 2006; Lang et al., 2006; Liu et al., 2007), and its induction by GCs is potentially a powerful anti-inflammatory mechanism (Clark, 2003; Clark and Lasa, 2003). Indeed, a variety of commonly prescribed GCs induced DUSP1 expression in alveolar macrophages in a manner roughly proportional to their anti-inflammatory efficacies (Zhao et al., 2005).

Macrophages derived from DUSP1<sup>-/-</sup> mice displayed an interesting partial GC-insensitivity (Abraham et al., 2006) (Figure 1). Dex inhibited the expression of IL-1 $\alpha$  mRNA strongly in DUSP1<sup>+/+</sup> but not at all in DUSP1<sup>-/-</sup> macrophages. In contrast the expression of CSF2 mRNA was equally strongly inhibited by dex in both DUSP1<sup>+/+</sup> and DUSP1<sup>-/-</sup> macrophages. Between these extremes, many pro-inflammatory genes (for example COX-2, TNF and IL-1 $\beta$ ) showed intermediate responses, dex-mediated suppression being impaired to some extent but not ablated in the absence of DUSP1. From these observations it can be concluded that 1) the expression of DUSP1 contributes importantly to the anti-inflammatory action of GCs on murine macrophages; 2) at least one additional, DUSP1-independent mechanism exists for the inhibition of inflammatory gene expression; 3) individual genes appear to be suppressed by both DUSP1-dependent and DUSP1-independent mechanisms. In a simple model of an acute inflammatory response, GCs exerted anti-inflammatory effects in wild type but not DUSP1<sup>-/-</sup> mice (Abraham et al.,

2006). Although these observations provide support for the hypothesis that DUSP1 is an important contributor to anti-inflammatory actions of GCs, several important questions remain unanswered.

LPS-induced lethal endotoxic shock in mice was dependent on p38 MAPK activation and could be inhibited by GCs (Bhattacharyya et al., 2007). Macrophage specific knockout of GR expression increased susceptibility to LPS-induced lethal endotoxic shock, which was associated with failure of GCs to induce DUSP1 and inhibit p38 MAPK activation (Bhattacharyya et al., 2007). This implies that the macrophage is an important site of action of GCs in one experimental model of acute inflammation, and that important anti-inflammatory effects are dependent on inhibition of p38 MAPK. Recent studies also implicate DUSP1 in the anti-inflammatory response to GCs in endothelial cells (Wadgaonkar et al., 2004; Furst et al., 2007), microglia (Zhou et al., 2007) and human airway smooth muscle cells (Issa et al., 2007). However, it is not known whether DUSP1 contributes to the anti-inflammatory effects of GCs on other cells such as mast cells, T lymphocytes or dendritic cells. Related to this, it will be important to establish whether ablation of the DUSP1 gene compromises the anti-inflammatory actions of GCs in complex models of immune-mediated inflammatory disease, and if so, in which cell types the expression of DUSP1 plays a critical role.

At least in macrophages DUSP1 was particularly important for GC-mediated suppression of inflammatory mRNAs that are stabilized by the p38 MAPK pathway (Abraham et al., 2006; Clark et al., 2003). Therefore dex may inhibit inflammatory gene expression in part by inducing DUSP1, inhibiting p38 MAPK and consequently destabilizing inflammatory mRNAs. However, this remains to be demonstrated, and other mechanisms may be involved. For example JNK is required for the phosphorylation and activation of c-Jun (Ip and Davis, 1998; Kracht and Saklatvala, 2002). GC-induced expression of DUSP1 is accompanied by a decrease in phosphorylation of nuclear c-Jun (Wu, Roth et al., 2006; Zhou et al., 2007), with consequences that may be indistinguishable from classical

transrepression of AP1. The p38 MAPK pathway can influence the transcriptional activation properties of NFκB (Vermeulen et al., 2003) or its recruitment to binding sites in promoters of pro-inflammatory genes (Saccani et al., 2002). A bacterially encoded dual-specificity phosphatase that targets p38 MAPK inhibits the transcriptional activation of certain NFκB-dependent promoters (Arbibe et al., 2007), and it is tempting to speculate that DUSP1 may have similar effects. The exact mechanisms by which GC-induced DUSP1 inhibits gene expression have not been elucidated.

In osteoblasts dex-induced expression of DUSP1 was associated with phosphatase-mediated inhibition of proliferation (Engelbrecht et al., 2003; Hulley et al., 2002). An involvement of DUSP1 in GC-induced osteoporosis has been postulated, but not yet proven. In adipocytes dex-induced DUSP1 expression was accompanied by impairment of insulin-induced glucose uptake (Bazuine et al., 2004), and DUSP1<sup>-/-</sup> mice displayed alterations of fat metabolism, manifesting as increased resistance to diet-induced obesity (Wu, Roth et al., 2006). These findings imply that upregulation of DUSP1 by GCs may contribute to metabolic dysregulation. Another important side effect, GC-induced cataract, was suggested to involve the induction of DUSP1 expression in lens epithelial cells (Gupta et al., 2007). Upregulation of DUSP1 by GCs appears to contribute to resistance of breast cancer cells to chemotherapeutic agents (Wu et al., 2004; Wu et al., 2005; Wu, Zou et al., 2006). These observations raise the possibility that both anti-inflammatory and unwanted effects of GCs could be mediated by a single molecule, expressed in different tissues.

### **Tristetraprolin.**

Tristetraprolin (TTP) is an mRNA destabilizing protein that recognizes specific transcripts and causes their rapid degradation via the recruitment of exonucleases (Carrick et al., 2004). A TTP knockout mouse strain has a complex phenotype with features of inflammatory arthritis, both of which are

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largely mediated by increased stability of TNF mRNA and increased expression of TNF protein (Carballo et al., 1998; Taylor et al., 1996). TTP recognizes the nonameric sequence UUAUUUAUU, present in the 3' untranslated region (UTR) of TNF mRNA (Brewer et al., 2004; Michel et al., 2003; Worthington et al., 2002). Many other mediators of immunity or inflammation are encoded by labile mRNAs that also have adenylate/uridylate-rich elements (AREs) in their 3' UTRs. COX-2, CSF2 and IL-2 genes are dysregulated in the absence of TTP (Carballo et al., 2000; Ogilvie et al., 2005; Phillips et al., 2004), and it seems likely that expression of other inflammatory mediators will also prove to be controlled by TTP. Inflammatory mRNAs can be stabilized via the phosphorylation and inactivation of TTP by MAPK-activated protein kinase 2, a kinase that is activated by p38 MAPK (Carballo et al., 2001; Chrestensen et al., 2004; Stoecklin et al., 2004). At least in cells of the myeloid lineage, this appears to be the principal mechanism for post-transcriptional regulation of inflammatory genes by the p38 MAPK pathway. Interestingly, DUSP1 mRNA contains two copies of the UUAUUUAUU sequence and DUSP1 expression is dependent on p38 MAPK in UV-stimulated HeLa cells (Lasa et al., 2002). However, our preliminary experiments suggest that p38 MAPK does not regulate DUSP1 expression at a post-transcriptional level, at least in HeLa cells (unpublished observations).

Expression of TTP mRNA was elevated by dex treatment of pulmonary epithelial A549 and BEAS-2B cells (Ishmael et al., 2007; Smoak and Cidlowski, 2006), primary human keratinocytes (Stojadinovic et al., 2006) or HeLa cells (our unpublished observations). GC-induced expression of TTP mRNA and protein in several rat tissues was confirmed by quantitative PCR and western blotting (Smoak and Cidlowski, 2006). siRNA-mediated reduction of TTP expression decreased the inhibitory effect of dex on expression of endogenous TNF mRNA or a reporter carrying the TNF 3' UTR (Smoak and Cidlowski, 2006). Importantly, inhibitory effects of GC (particularly on chemokine gene expression) were impaired in TTP<sup>-/-</sup> fibroblasts (Ishmael et al., 2007).



The induction of TTP may contribute to the post-transcriptional inhibitory effects of GCs described above. However, it should be noted that dex inhibited the LPS-induced expression of TTP mRNA and protein in macrophages (Jalonen et al., 2005), and had no effect on PMA/PHA-induced TTP protein expression in human peripheral blood mononuclear cells (Bergmann et al., 2004). The control of TTP expression by pro-inflammatory stimuli and GCs needs further investigation. It will also be interesting to determine whether anti-inflammatory actions of GCs are impaired in TTP<sup>-/-</sup> macrophages and other cells. There is potential for cooperative effects of two different GC-induced factors. GCs could induce the expression of TTP and at the same time increase its activity by upregulating DUSP1 and inhibiting p38 MAPK. As yet, this hypothesis remains unexplored.

### **Mast cell signaling effectors.**

Activation of mast cells through engagement of Ig receptors initiates a complex cell signaling cascade that includes stimulation of src-like tyrosine kinases, activation of PI3K-Akt and Raf-MEK-ERK pathways, and mobilization of intracellular calcium stores. These coordinately regulate not only the upregulation of pro-inflammatory gene expression, but also the rapid release of preformed inflammatory mediators from storage granules. In allergic diseases like asthma GCs can inhibit both immediate and delayed responses to antigen exposure (Kassel and Cato, 2002). In response to dex, RBL-2H3 cells (a rat mast cell line) upregulated DUSP1 (Kassel et al., 2001) Dok-1 (downstream of tyrosine kinase 1) (Hiragun et al., 2005) and SLAP (src-like adaptor protein) (Hiragun et al., 2006). Each of these is an inhibitor of signaling pathways critical to mast cell activation. DUSP1 (see above) was implicated in dex-mediated inhibition of mast cell ERK signaling (Kassel et al., 2001). Dok-1 can associate with the Ras GTPase-activating protein and inhibit activation of Raf and ERK. Overexpression of Dok-1 partially mimicked effects of dex on the ERK pathway, whereas RNAi-mediated reduction of Dok-1 levels enhances inflammatory responses of mast cells (Hiragun et al., 2005). Overexpression of SLAP mimicked several effects of dex, inhibiting antigen-induced

phosphorylation of the src-like tyrosine kinase syk, activation of ERK, calcium mobilization and degranulation. Conversely, siRNA-mediated reduction of SLAP levels impaired the effects of dex on tyrosine kinase activation and calcium mobilization (Hiragun et al., 2006).

It is striking that mast cells respond to GCs by upregulating a number of negative regulators of signaling. These genomic responses to GCs are generally quite slow, and are probably most significant in the context of sustained GC exposure. There is not yet compelling evidence as to which negative regulators are important for the anti-inflammatory effects of GCs *in vivo*, since GC effects on mast cell function have not been described in DUSP1<sup>-/-</sup>, Dok-1<sup>-/-</sup> (Di Cristofano et al., 2001; Yamanashi et al., 2000) or SLAP<sup>-/-</sup> (Sosinowski et al., 2000) mice. RNAi knockdown of SLAP reduced the effects of dex on signaling pathways but did not alter dex-mediated inhibition of mast cell degranulation (Hiragun et al., 2006). Possibly GC-induced inhibitors of signaling serve redundant functions in the inhibition of mast cell responses. It has not been reported whether GCs induce expression of Dok-1 or SLAP in cells other than mast cells.

### **Glucocorticoid Inducible Leucine Zipper.**

GCs upregulate glucocorticoid inducible leucine zipper (GILZ) expression in T cells (Asselin-Labat et al., 2004; D'Adamio et al., 1997; Mittelstadt and Ashwell, 2001; Riccardi et al., 2001), mast cells (Godot et al., 2006), eosinophils (Arthaningtyas et al., 2005), epithelial (Bhalla et al., 2006; Chivers et al., 2006; Eddleston et al., 2007; Soundararajan et al., 2005; Wang et al., 2004), and myeloid cells (Berrebi et al., 2003; Cohen et al., 2006; Ehrchen et al., 2007). Relatively high basal GILZ expression has also been recorded in brain, lung and skeletal muscle (Cannarile et al., 2001). GILZ is a 137 amino acid protein with a central leucine zipper (LZ) dimerization domain. Unlike other LZ proteins it is not known to bind directly to DNA, but interacts with NFκB and impairs its transactivation function (Ayroldi et al., 2001; Berrebi et al., 2003; DiMarco et al., 2006; Eddleston et al., 2007; Riccardi et al.,

2001). GILZ may similarly inhibit the function of AP1 (Mittelstadt and Ashwell, 2001) and transcription factors that recognise the conserved lymphokine element 0 (CLE0), a cis-acting element required for efficient expression of IL-5 (Arthaningtyas et al., 2005). Overexpression of GILZ in transfected T cells downregulates IL-2 and Fas ligand, protecting against activation-induced cell death (Ayroldi et al., 2001; Ayroldi et al., 2002; D'Adamio et al., 1997). T cell-directed overexpression of GILZ in a transgenic mouse strain mimics some of the effects of GCs on the immune system, for example inhibition of activation-induced apoptosis (Delfino et al., 2006) and skewing of T cell differentiation towards the Th2 subtype (Cannarile et al., 2006). Therefore, some of the immune modulatory effects of GCs may be mediated in part by the expression of GILZ in T cells.

GILZ overexpression in a monocytic cell line can downregulate chemokines and activation markers (Berrebi et al., 2003), suggesting an anti-inflammatory function in the myeloid compartment. Immunomodulatory effects of GCs on dendritic cells were mimicked by the overexpression of GILZ and blocked by siRNA-mediated knockdown of GILZ (Cohen et al., 2006). RNA interference experiments also supported a role for GILZ in the inhibition of CXCL8 expression in endothelial cells (Eddleston et al., 2007). On the other hand, GILZ stimulated rather than suppressed the expression and activity of the epithelial sodium channel  $\alpha$  (ENaC $\alpha$ ) (Bhalla et al., 2006; Soundararajan et al., 2005), possibly via inhibition of the ERK pathway (Ayroldi et al., 2002; Soundararajan et al., 2005). ENaC $\alpha$  expression is thought to contribute to GC-induced hypertension (Fuller et al., 2000; Itani et al., 2002; Sayegh et al., 1999; Stokes and Sigmund, 1998). This raises the interesting possibility that a GC-induced gene could contribute to both anti-inflammatory and harmful effects in a single cell type. Unfortunately no knockout has so far been generated to provide conclusive proof of the suggested functions of GILZ in myeloid or other cells. In transfected cells the induction of GILZ by GC was dependent on dimerization of GR (Rogatsky et al., 2003), although GILZ expression in the GR<sup>dim</sup>

mouse has not yet been described. GR interacted *in vivo* with a distal 5' region of the GILZ locus that contains four divergent GRE sequences (Wang et al., 2004). Both these GR binding sites and FoxO3 binding sites (Asselin-Labat et al., 2005) have been implicated in the transcriptional regulation of GILZ by GCs.

### **IL-10.**

IL-10 is a pleiotropic anti-inflammatory and immunomodulatory cytokine, which is expressed by several haematopoietic cell types (Grutz, 2005; Williams et al., 2004). Induction of IL-10 expression has been suggested to contribute to the anti-inflammatory properties of GCs in rheumatoid arthritis (Verhoef et al., 1999), asthma (Karagiannidis et al., 2004; Stelmach et al., 2002), multiple sclerosis (Gayo et al., 1998) and cardiac bypass surgery (Tabardel et al., 1996). In these complex clinical settings the cellular source of IL-10 is often unclear. *In vitro*, purified monocytes or macrophages but not B or T cells expressed IL-10 in response to GC (Ehrchen et al., 2007; Hodge et al., 1999; Marchant et al., 1996; Mozo et al., 2004). Differentiation of dendritic cells (dc) in the presence of GC gave rise to a dc population with decreased cell surface costimulatory molecule expression and increased capacity to express IL-10 when stimulated (de Jong et al., 1999; Dong et al., 2003; Duperrier et al., 2005; Matyszak et al., 2000; Piemonti et al., 1999; Rea et al., 2000; Rozkova et al., 2006). Antigen presentation by dc in the absence of a strong costimulatory signal can drive expansion of T cells that themselves express IL-10 and have immunosuppressive “regulatory” properties (Dong et al., 2003). Hence both direct and indirect pathways for the induction of IL-10 expression may contribute to immunosuppressive effects of GCs.

The perfect palindromic sequence AGAACAGCTGTTCT in the human IL-10 promoter resembles a GRE, although with two rather than three nucleotides between the TGTCT half sites. This sequence is quite well conserved in the murine IL-10 promoter, but it is not yet known whether either element

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contributes to GC-mediated upregulation of IL-10. Expression of IL-10 is acutely dependent on p38 MAPK (Foey et al., 1998; Koprak et al., 1999; Ma et al., 2001) and dysregulated in DUSP1<sup>-/-</sup> macrophages (Abraham et al., 2006; Chi et al., 2006; Hammer et al., 2006; Zhao et al., 2006). There may exist a pathway for GC-mediated downregulation of IL-10 expression via the induction of DUSP1 and inhibition of p38 MAPK, but this has not been conclusively demonstrated (Abraham et al., 2006). The regulation of IL-10 is not well understood, and may be determined by the outcome of both positive and negative GC-induced mechanisms and other signaling pathways.

### **Annexin A1.**

Annexin A1 (AnxA1) is a member of a family of calcium-dependent phospholipid binding proteins that provide a functional link between calcium signalling and membrane organization (Gerke et al., 2005). Members of this family are postulated to play roles in the establishment or maintenance of membrane microdomains and communication between membrane compartments (Gerke et al., 2005). AnxA1 was first identified as a factor that was released from GC-stimulated cells *in vitro* or *in vivo*, and exerted anti-inflammatory effects mirroring those of GCs. Most notably it inhibited release of pro-inflammatory prostaglandins, and impaired leukocyte extravasation at sites of inflammation (Chatterjee et al., 2005; de Coupade et al., 2003; Gavins et al., 2003; Lim and Pervaiz, 2007; Liu et al., 2005; Parente and Solito, 2004). AnxA1 is relocalized from the cytoplasm to the plasma membrane in response to a variety of agonists including GCs, and can be externalized or secreted via an uncharacterized mechanism. The N-terminus of AnxA1, which is distinct from those of other annexins, is thought to exert anti-inflammatory effects by signalling through members of the formyl peptide receptor (FPR) family, in particular FPRL1 (Ernst et al., 2004; Gavins et al., 2003; Hayhoe et al., 2006; John et al., 2007; Perretti et al., 2001; Walther et al., 2000). This cell surface receptor also recognizes as a ligand lipoxin A<sub>4</sub>, a prostanoid involved in the resolution of inflammation.

Neutralizing antibodies against AnxA1 impaired the anti-inflammatory effects of GCs on carageenin-induced paw oedema (Duncan et al., 1993), ischaemia-reperfusion injury (D'Amico et al., 2000) and adjuvant-induced arthritis (Yang et al., 1999). Inflammatory responses of an AnxA1 null mouse strain were exaggerated (Chatterjee et al., 2005; Hannon et al., 2003; Warne et al., 2006; Yang et al., 2004), and cells derived from these mice overexpressed COX-2, IL-1 $\beta$  and -6 in response to pro-inflammatory stimuli (Croxtall et al., 2003; Damazo et al., 2006; Hannon et al., 2003; Yang et al., 2006; Yona et al., 2004; Yona et al., 2005), suggesting that AnxA1 is an endogenous inhibitor of inflammatory responses (Perretti and Gavins, 2003). It should be noted here that cells lacking AnxA1 also showed differences of morphology (Croxtall et al., 2003) and phagocytic properties (Yona et al., 2004), which might be indicative of altered membrane physiology. Importantly, suppressive effects of GCs on carageenin-induced oedema, zymosan-induced peritonitis and antigen-induced arthritis were impaired in AnxA1<sup>-/-</sup> mice (Hannon et al., 2003; Yang et al., 2004). GCs failed to inhibit the expression of IL-6 and COX-2 in lung fibroblasts lacking AnxA1 (Hannon et al., 2003; Yang et al., 2006). In part this may be explained by the failure of AnxA1<sup>-/-</sup> cells to express DUSP1 and inhibit p38 MAPK signalling (Yang et al., 2006).

From these observations it has been argued that AnxA1 is a mediator of certain anti-inflammatory actions of GCs. It also appears to contribute to at least one of the important side effects of GCs, the suppression of the HPA axis and consequent adrenal insufficiency (Buckingham et al., 2006). To clarify whether externalization of AnxA1 is crucial to its role in GC responses, it would be of interest to determine whether anti-inflammatory actions of GCs are impaired in FPRL1 null mice. Although AnxA1 mRNA expression is upregulated by GCs this response tends to be quite slow and modest (less than two-fold) (Solito et al., 2003). It may have more to do with the homeostatic maintenance of cellular AnxA1 levels than the rapid anti-inflammatory response to GCs.

**IL-1 receptor type II.**

The non-signaling type II IL-1 receptor (IL-1RII) is thought to function as a decoy receptor that inhibits cellular responses to IL-1 (Neumann et al., 2000). GC-induced upregulation of IL-1RII has been described in a number of studies (Brown et al., 1996; Colotta et al., 1993; Ehrchen et al., 2007; Pousset et al., 2001) and may contribute to inhibition of a very specific subset of (IL-1-mediated) inflammatory responses. This decoy receptor upregulation must be seen in the context of GC-induced upregulation of several signaling receptors, such as Toll-like receptors, that can potentially transmit pro-inflammatory signals (Rozkova et al., 2006; Schleimer, 2004).

 **$\beta$  adrenergic agonist receptors.**

$\beta$  adrenergic agonists cooperate with GCs to exert anti-inflammatory effects in asthma (Barnes, 2002; Hancox, 2006). GCs can upregulate expression of  $\beta$ 1 and  $\beta$ 2 adrenergic receptors (Collins et al., 1988; Mak et al., 1995; Tseng et al., 2001), potentially providing a rather indirect anti-inflammatory mechanism by sensitizing target cells to additional anti-inflammatory stimuli.

**CONCLUSIONS.**

The side effects of GCs involve changes in expression of countless genes in several different tissues. Although the underlying molecular mechanisms are “complex, distinct, and frequently only partly understood” (Schacke et al., 2002), there is a growing recognition that both activation and inhibition of gene expression by GR are involved. We would argue that exactly the same is true of the anti-inflammatory effects of GCs (see Figure 2). As discussed above, GCs can exert anti-inflammatory effects at both transcriptional and post-transcriptional levels, via both transrepression and transcriptional activation. The GC-induced expression of DUSP1, GILZ, Dok-1 or SLAP can interfere with signaling pathways that are activated by pro-inflammatory stimuli, and hence block pro-

inflammatory gene expression. The function of NF $\kappa$ B may be inhibited via the upregulation of I $\kappa$ B $\alpha$ , GILZ and perhaps DUSP1, with consequences that are difficult to distinguish from classical transrepression without careful case-by-case analysis. In a similar fashion, DUSP1 expression and JNK suppression may contribute to GC-mediated inhibition of AP1 function. Induction of DUSP1 and TTP gene expression contributes to anti-inflammatory effects of GCs at the post-transcriptional level. This is emerging as an important component of the anti-inflammatory armoury of GCs, but is absent from models based on transrepression, which view changes in mRNA abundance as equivalent to changes at the transcriptional level. Secreted or externalized proteins such as IL-10, AnxA1 and IL-1RII are also likely to contribute to the anti-inflammatory response to GCs. Other GC-induced anti-inflammatory mediators have been omitted from this review for reasons of space, and it is probable that yet more will come to light. It is difficult to argue that a single GC-induced factor plays an indispensable role in the anti-inflammatory response, but still harder to argue that the positive regulation of gene expression by GCs is irrelevant to their anti-inflammatory action.

In fact the side effects of GCs can be regarded as physiological responses that have evolved through natural selection but are driven to extremes by abnormally sustained high concentrations of potent, synthetic GCs in the bloodstream. For example the induction of gluconeogenesis is, on one hand, a component of the healthy response to physical or psychological stress and, on the other, an underlying cause of GC-induced diabetes mellitus. The distinction between anti-inflammatory effects and side effects seems rather artificial in the sense that both are “on-target” GC responses. Given the extraordinary array of mechanisms through which GCs can modulate gene expression, why should we expect distinct mechanisms to be deployed along the lines of clinical convenience or inconvenience? It is difficult to provide an account of evolutionary pressures that would lead to such an outcome.



A problem with the current paradigm is that many rather simple phenomena have been made to stand as representative of biological processes that are vastly more complex. Reporter constructs containing multimerized, idealized palindromic GREs are considered to represent GC-induced gene expression, although it now seems clear that straightforward GRE-mediated transcriptional regulation accounts for a small fraction of the GC response. Alternatively, model genes such as TAT and PEPCK are used as illustrations of GC-induced transcriptional responses. These genes are regulated by GCs in a more complex manner than originally believed, but other GC targets are controlled by dissimilar, equally complex and extremely varied mechanisms. Again, induction of TAT and PEPCK is considered representative of GC-induced side effects, although such side effects are poorly understood and highly diverse in mechanism. The argument that GC-induced gene expression does not contribute to the anti-inflammatory response is largely based on the failure of GR<sup>dim</sup> to induce TAT and PEPCK, yet these genes are not putative anti-inflammatory mediators. As surrogate markers for cellular responses to GCs, TAT and PEPCK are employed far beyond their utility. Furthermore, models that do not adequately reflect the complexity of GC action are likely to have poor predictive power. Simple drug screens based on idealized reporter constructs or a few representative GC targets seem unlikely to succeed in identifying compounds that clearly discriminate between the positive and negative regulatory properties of GR. For example a first generation “dissociated” GC failed to activate a GRE-dependent reporter construct but strongly induced expression of at least three putative anti-inflammatory mediators, AnxA1, GILZ and DUSP1 (Chivers et al., 2006; Janka-Junttila et al., 2006).

There is a strong case for developing a more comprehensive model of anti-inflammatory actions of GCs, in which the importance of GC-induced gene expression is recognized and both transcriptional and post-transcriptional mechanisms are featured. The relative importance of transcriptional and post-transcriptional effects on a given gene may be dependent on the cellular context, activation status and time at which GCs are present. As illustrated schematically in Figure 2, the molecular mechanisms

involved in the side-effects and anti-inflammatory actions of GCs may prove to be inextricably tangled. An important new concern is that upregulated factors may prove to contribute to both anti-inflammatory and unwanted effects of GCs. DUSP1 is a putative example of such a two-edged mediator of GC responses (represented by gene B in Figure 2B). What would all of this imply for the development of safer anti-inflammatory therapies? As argued elsewhere (Hiragun et al., 2006; Smoak and Cidlowski, 2006), novel GR agonists that fail to activate gene expression may lack an important component of anti-inflammatory action. On the other hand, attempts to exert anti-inflammatory effects by modulating expression of DUSP1 or other factors risks causing side effects unless exquisite tissue targeting and control can be achieved. In summary, there are unlikely to be effective short-cuts to the discovery of truly dissociated GR agonists and it is debatable whether such compounds, if they can be identified, will yield the predicted clinical benefits.

**Acknowledgements.**

The work of Andy Clark's lab is supported by a programme grant from the Medical Research Council (UK). The author is grateful to Sonya Abraham, Rob Newton, Jeremy Saklatvala and Cristiana Stellato for helpful discussions during the writing of this review.

Accepted Manuscript

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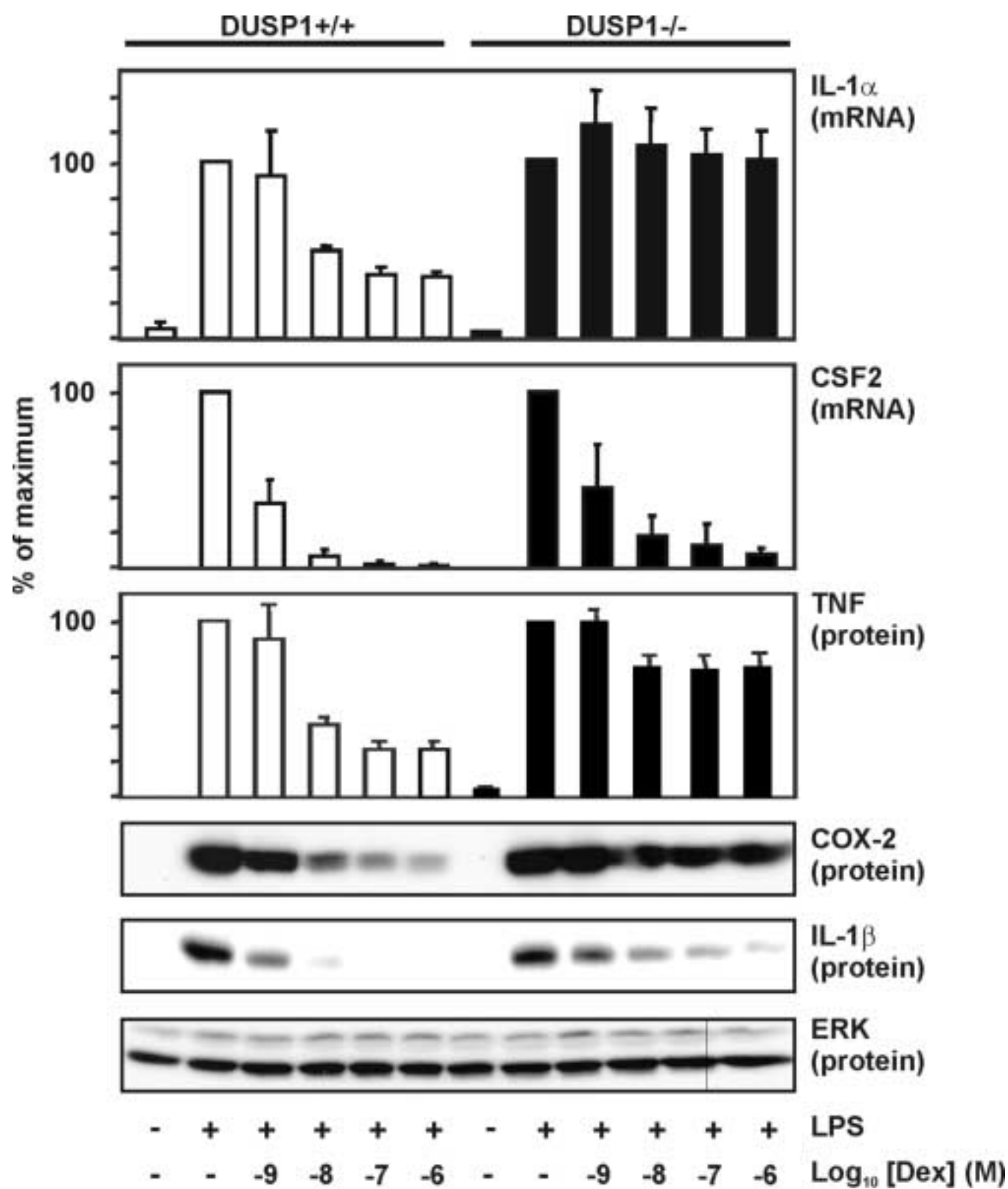
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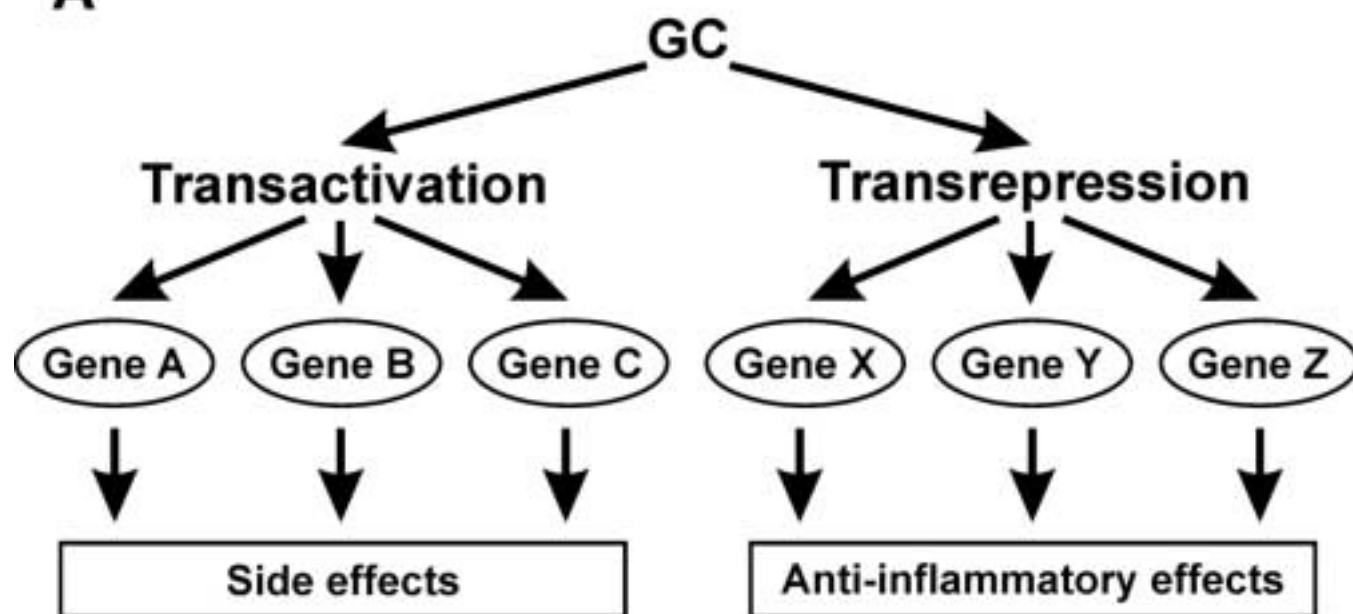
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**Figure 1. DUSP1 contributes to anti-inflammatory effects of dex on murine macrophages.** Bone marrow-derived macrophages from age- and sex-matched DUSP1<sup>+/+</sup> and DUSP1<sup>-/-</sup> mice were pretreated with 1 nM to 1  $\mu$ M dex for 4 h then stimulated with 10 ng/ml LPS for 4 h. IL-1 $\alpha$  and CSF2 mRNAs were quantified by ribonuclease protection assay; TNF protein expression was measured by ELISA; COX-2, IL-1 $\beta$  and ERK proteins in whole cell lysates were detected by western blotting. Adapted from (Abraham et al., 2006).

**Figure 2. Mechanisms of side effects and anti-inflammatory effects of GCs.** In a simple model (A) GCs transactivate genes A, B and C, which all contribute to GC side effects. GCs transrepress genes X, Y and Z, which all contribute to anti-inflammatory effects. In an amended model (B) there is no clear mechanistic separation between anti-inflammatory effects and side effects. Transactivation of gene C contributes to the anti-inflammatory response, whilst transrepression of gene X contributes to side effects. Transactivation of gene B mediates both anti-inflammatory effects and side effects, depending on the context in which it is expressed. Similarly, transrepression of gene Y can have both therapeutic and harmful consequences.





**A****B**